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Immunological Tolerance to a Pancreatic Antigen as a Result of Local Expression of TNFα by Islet β Cells

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Summary

Recent experiments have suggested that tumor necrosis factor α (TNFα) can down-regulate islet-specific T cells and prevent the development of autoimmune diabetes. Here we demonstrate that transgenic mice expressing both TNFα and the Leishmania major LACK antigen in the pancreas (RIP-TNFα/RIP-LACK) exhibit an impaired ability to mount a $CD4^+$ T cell response against LACK. In addition, peripheral $CD4^+$ T cells from TCR transgenic mice (TCR-LACK/RIP-TNFα/RIP-LACK) produced reduced interleukin-2 but elevated levels of T helper 2 cytokines in response to LACK peptide in vitro. Taken together, our data suggest that TNFα may act in vivo to modulate a potentially damaging self-reactive T cell response by inducing tolerance to pancreatic antigens.

Introduction

Tumor necrosis factor α (TNFα) is a potent inflammatory mediator that is involved in the pathological processes found in a number of autoimmune and infectious disease states (Ruddle et al., 1990; Jacob, 1992; Lane et al., 1992; Vassalli, 1992). TNFα is produced predominantly by activated macrophages, but also by other cell types such as mast cells, neutrophils, and $CD4^+$ T helper 1 (Th1) cells (Vassalli, 1992). The ubiquitous expression of TNF receptors suggests that TNFα may mediate a number of biological activities in a wide variety of cells and tissues. Besides its well-documented proinflammatory role, TNFα has been shown to promote T cell proliferation in vitro (Shalaby et al., 1988; Yokota et al., 1988), to prevent T cell deletion induced by superantigens (Vella et al., 1995), and to be critically required for germinal center formation following immunization (Pasparakis et al., 1996). All of these functions, which contribute to the establishment, maintenance, or amplification of specific immune responses, may also lead to tissue injury in pathological situations.

This picture is further complicated, however, by growing evidence that TNFα can also down-regulate immune responses and/or prevent the development of immune-mediated pathology. Thus, TNFα induces the apoptotic death of T cell blasts in vitro (Sarin et al., 1995) and contributes with CD95–CD95L interactions to the peripheral death of activated T cells in vitro (Zheng et al., 1995) and in vivo (Sytwu et al., 1996). In agreement with a possible role for TNFα in the prevention of immune-mediated pathology, mice lacking TNF receptor 1 develop chronic inflammatory lesions when infected with Leishmania major (Vieira et al., 1996) and exhibit an accelerated autoimmune condition when crossed to the *lpr* strain (Zhou et al., 1996).

The pleotropic nature of TNFα has been well documented in nonobese diabetic (NOD) mice, which spontaneously develop autoimmune diabetes, a chronic T cell–mediated disease that is characterized by lymphocytic infiltration of the pancreatic islets (insulitis) and by the selective destruction of insulin-producing β cells (Tisch and McDevitt, 1996). Experiments demonstrating that TNFα mRNA is produced in situ in inflamed islets in NOD mice have suggested that TNFα plays a pivotal role in the disease process (Held et al., 1990). Consistent with this hypothesis, treatment of newborn NOD mice with recombinant TNFα or with neutralizing anti-TNFα monoclonal antibodies (MAbs) or soluble TNF receptor leads to earlier onset or complete prevention of disease, respectively (Yang et al., 1994; Hunger et al., 1997).

TNFα may be directly involved in the destruction of β cells themselves or may contribute to the diabetogenic process through the recruitment of autoreactive T cells to the inflamed islets. The generation of transgenic mice that express TNFα in pancreatic β cells (RIP-TNFα mice) has demonstrated that at least the latter may be true, since these mice develop increasingly massive lymphocytic infiltration within the islets, apparently resulting from the induction of endothelial changes (Higuchi et al., 1992; Picarella et al., 1993). However, recruitment of a cellular infiltrate is not sufficient for the induction of the disease, since RIP-TNFα transgenic mice do not become diabetic, even after perfusion with other cytokines, mild damaging of β cells, or transgenic expression of a viral antigen on β cells (Higuchi, et al., 1992). Moreover, RIP-TNFα mice generated on the NOD background do not progress from insulitis to diabetes, suggesting that TNFα may actually inhibit one of the late phases of the disease process (Grewal et al., 1996). Indeed, these data are in agreement with earlier studies that demonstrated that TNFα, when administered to NOD mice after

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4 weeks of age, can dramatically prevent the onset of the disease (Satoh et al., 1989; Jacob et al., 1990). It is not known how spontaneous or experimentally induced local expression of TNFα in islets could prevent or delay the development of diabetes. However, this protective effect of transgenic TNFα production in NOD mice was found to correlate with a lack of T cell responsiveness to islet antigens, a process that occurs spontaneously in NOD mice.

To elucidate how local expression of TNFα can affect T cell responses directed to islet antigens, we generated double-transgenic mice by crossing RIP-TNFα mice (Picarella et al., 1993) to transgenic mice in which the expression of the L. major LACK antigen (Mougneau et al., 1995) was restricted to pancreatic β cells. We found that these mice failed to mount a T cell response to LACK after immunization and also developed depressed LACK-specific antibody responses when compared to single-transgenic RIP-LACK mice or negative littermates. This phenomenon was analyzed further by breeding these mice with TCR-LACK transgenic mice that express a TCR transgene specific for a class II–restricted LACK peptide. Taken together, our data suggest that local expression of TNFα can lead to the development of tolerance to pancreatic antigens, which may account for the previously reported capacity of this cytokine to prevent the development of autoimmune disease.

Results

Generation of Transgenic Mice Expressing the L. major LACK Antigen in Pancreatic β Cells

To determine how the local expression of TNFα would affect T cell responses to pancreatic antigens, we bred RIP-TNFα mice to another transgenic strain in which the expression of the L. major cytoplasmic LACK antigen was directed to the β cells (RIP-LACK). RIP-LACK founders were crossed to BALB/c mice, and offspring were monitored for LACK expression using reverse transcription polymerase chain reaction (RT-PCR). The expression of LACK mRNA was detected in purified pancreatic islets but not in any other organs examined, including thymus, liver, spleen, and kidneys (Figure 1). One RIP-LACK founder was backcrossed to BALB/c mice for five generations, and the colony was subsequently expanded. RIP-LACK mice were monitored for more than 1 year by measurement of urine glucose, and none of ten mice developed diabetes (data not shown). In addition, examination of pancreatic sections from adult RIP-LACK mice did not reveal any abnormality or evidence of insulitis (data not shown).

Peripheral Tolerance to LACK Occurs in RIP-LACK/RIP-TNFα Double-Transgenic Mice

In contrast to other transgenic strains in which defined antigens were expressed by islet β cells (Lo et al., 1992; Förster et al., 1995), RIP-LACK mice were not tolerant to LACK. Thus, following immunization with LACK recombinant protein in complete Freund's adjuvant (CFA), lymph node T cells from RIP-LACK mice secreted similar levels of interferon-γ (IFNγ) and interleukin-5 (IL-5) in response to LACK in vitro when compared to cells from

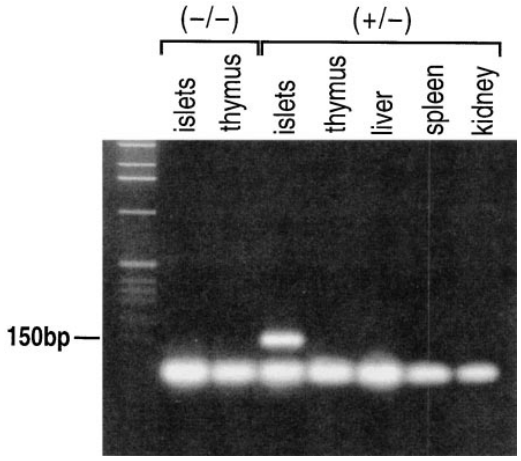

Figure 1. Expression of LACK mRNA Is Restricted to Pancreatic Islets in RIP-LACK Transgenic Mice

The presence of LACK-specific mRNA was examined in transgenic (+/−) and nontransgenic (−/−) mice by amplifying cDNA made from the indicated tissues using LACK-specific primers.

nontransgenic littermates (Figures 2A and 2B). Even though RIP-LACK mice were not tolerant to LACK, they did not develop diabetes, either spontaneously or following infection with L. major or immunization with LACK in CFA (data not shown).

To determine how the transgenic expression of TNFα in islet β cells would affect immune responses to a defined pancreatic antigen, we crossed RIP-LACK to RIP-TNFα transgenic mice. Surprisingly, when these mice were immunized with LACK, lymph node T cells from double-transgenic mice produced less IFNγ and IL-5 in response to LACK in vitro than cells from RIP-TNFα or RIP-LACK single-transgenic mice, indicating that these mice were tolerant to LACK (Figures 2A and 2B). These results were confirmed when we directly measured the numbers of IL-5– and IFNγ-secreting cells in the draining lymph nodes of immunized mice using a sensitive ELISPOT assay (Figures 3A and 3C). The number of IL-4–secreting cells was also reduced in double-transgenic RIP-LACK/RIP-TNFα mice as compared to that in mice from the other three groups (Figure 3B). In agreement with these results, reduced amounts of LACK-specific immunoglobulin G1 (IgG1) and IgG2a were found in the serum of immunized RIP-LACK/RIP-TNFα double-transgenic mice compared to the sera of RIP-LACK or RIP-TNFα transgenic mice or negative littermates (Figure 4). The observed LACK-specific tolerance found in RIP-LACK/RIP-TNFα mice did not appear to be the result of general immunosuppression, because mice from the four different groups all mounted comparable responses when immunized with ovalbumin (Figures 2C and 3D).

LACK-Reactive CD4+ T Cells Are Tolerized Prior to Immunization

Two different mechanisms could explain the impaired ability of double-transgenic RIP-LACK/RIP-TNFα mice to mount LACK-specific immune responses following immunization with LACK. One possibility could be that LACK-reactive cells are activated in the draining lymph

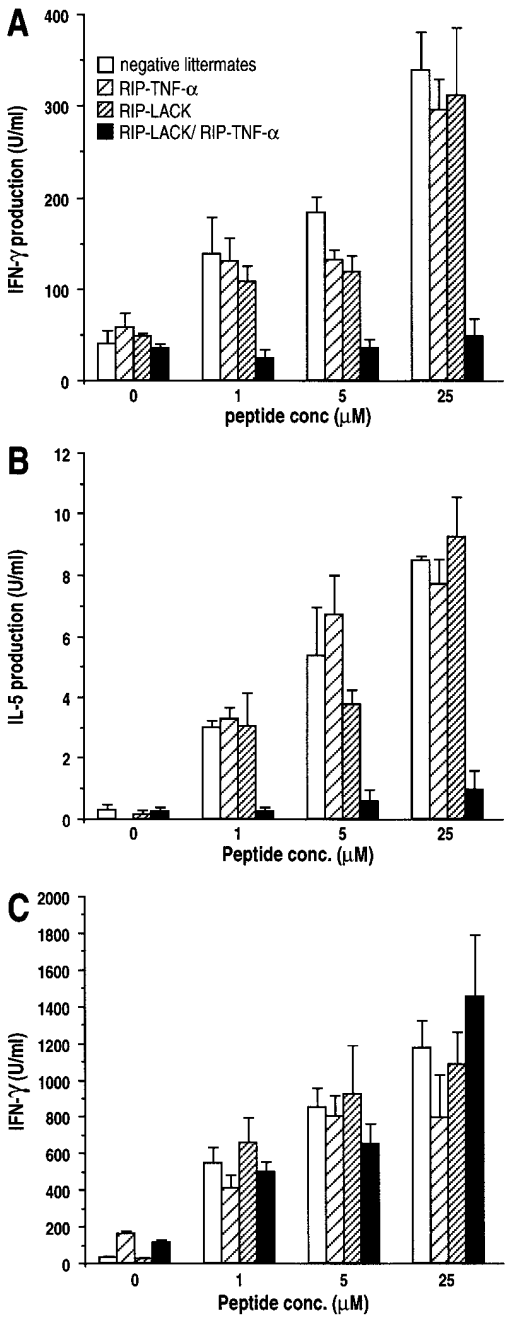

Figure 2. LACK-Specific Cytokine Responses Are Impaired in RIP-LACK/RIP-TNFα Transgenic Mice

RIP-LACK and RIP-TNFα single-transgenic mice, RIP-LACK/RIP-TNFα double-transgenic mice, and negative littermates were immunized with 50 μg of LACK or ovalbumin in CFA. Ten days after immunization, draining lymph node cells were cultured for 72 hr in the presence of medium alone or with the indicated concentrations of the LACK or ovalbumin peptides. Cytokine production was determined by ELISA for (A) IFNγ and (B) IL-5 in response to LACK and (C) IFNγ in response to ovalbumin. There was no detectable IL-4 in any of the samples analyzed. Data are means ± SEM of duplicates from three individual mice per group and are representative of four experiments.

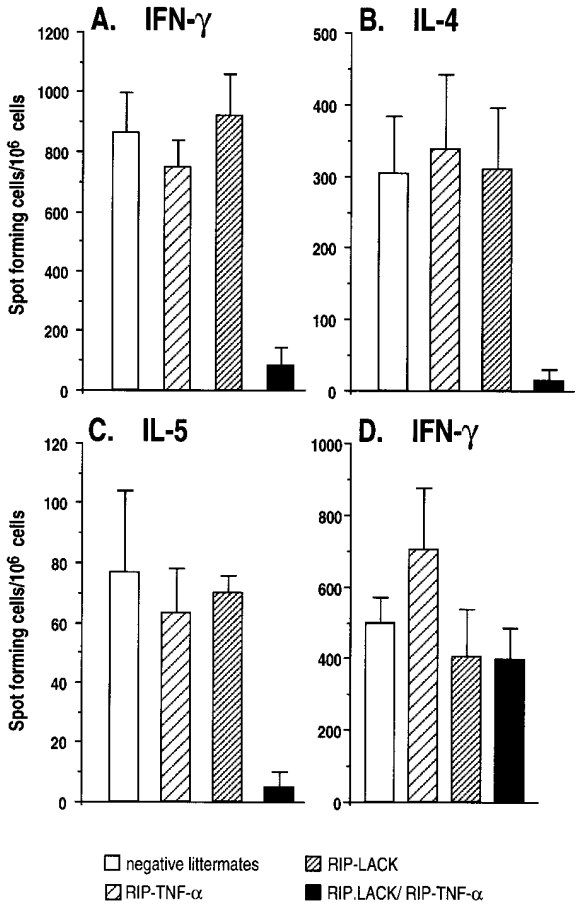

Figure 3. The Draining Lymph Nodes of Immunized RIP-LACK/RIP-TNFα Transgenic Mice Contain Reduced Numbers of LACK-Reactive Th1- and Th2-Like Cells

Lymph node cells from the indicated mice were incubated with an optimal concentration (25 μM) of LACK or ovalbumin peptide for 72 hr. Live cells were recovered and transferred by serial dilution to precoated 96-well microtiter plates. After 24 hr, cells were removed and spots were visualized using biotinylated detecting MAbs before the number of spots was determined. Data are numbers of (A) IFNγ–, (B) IL-4–, and (C) IL-5–producing cells in response to LACK and (D) IFNγ–producing cells in response to ovalbumin. Data are means ± SEM for three individual transgenic mice per group.

nodes following immunization and then migrate to the pancreas, where a second encounter with LACK in the presence of TNFα leads to their tolerization or prevents them from returning to the lymph nodes. Alternatively, local expression of TNFα and LACK in the pancreas could alter the phenotype of LACK-reactive cells prior to immunization, a phenomenon that could then be visualized when the mice are immunized with LACK. To determine which of these two hypotheses is correct, we adoptively transferred B cell–depleted spleen cells from single RIP-TNFα or double RIP-LACK/RIP-TNFα transgenic mice into nude mice. Cells from IE-LACK transgenic mice that were tolerant to LACK as the result of transgenic expression of this protein in the thymus were also used as a control. Nude recipients that received T cells from double RIP-TNFα/RIP-LACK or single IE-LACK transgenic mice exhibited an impaired ability to mount a LACK-specific T cell response following immunization, in contrast to nude mice that received T cells from RIP-TNFα single-transgenic mice. Thus, lymph node cells from mice that received T cells from

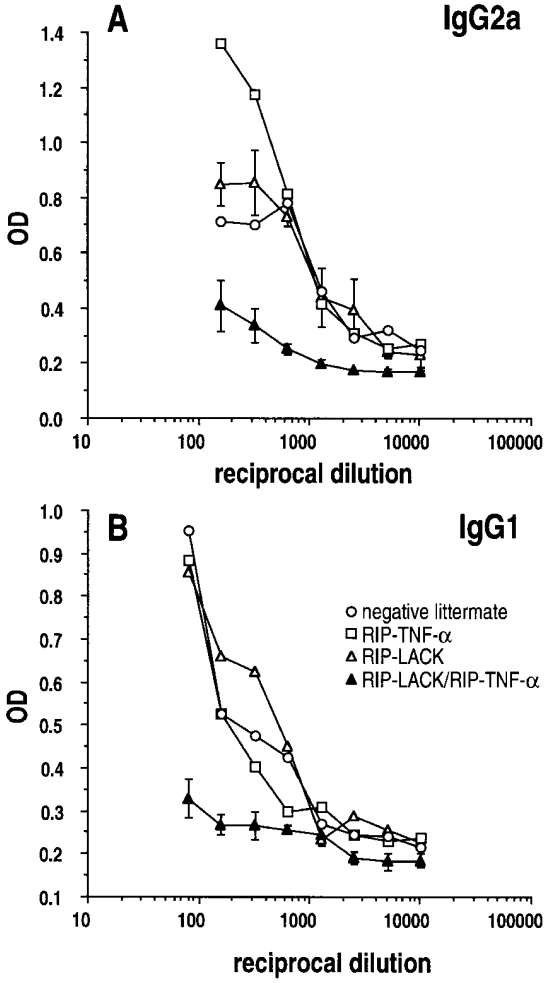

Figure 4. LACK-Specific Antibody Responses Are Impaired in RIP-LACK/RIP-TNFα Transgenic Mice

Mice from the four indicated groups were immunized with 50 μg of LACK in CFA. Ten days after immunization, the presence of serum IgG2a (A) and IgG1 (B) antibodies from individual mice was determined by ELISA. Data are means ± SEM for three individual transgenic mice per group.

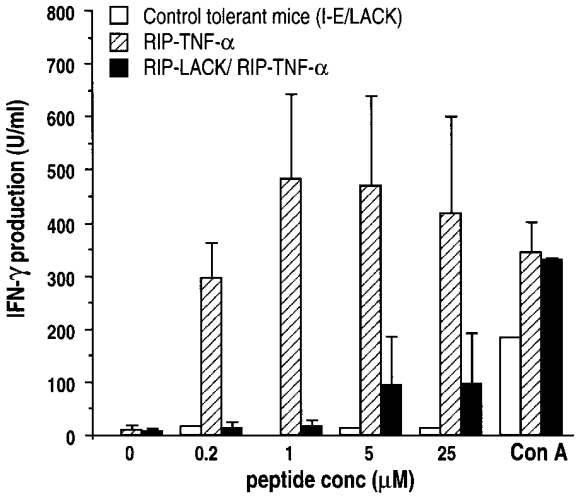

Figure 5. LACK-Specific Cytokine Responses Are Impaired in Nude Mice That Have Been Adoptively Transferred with T Cells from RIP-LACK/RIP-TNFα Transgenic Mice

Nude mice were injected intraveneously with B cell–depleted spleen cells from RIP-TNFα or RIP-LACK/RIP-TNFα mice or from IE-LACK mice, which are tolerant to LACK as the result of the transgenic expression of LACK in the thymus. One week after adoptive transfer, mice were immunized with 50 μg of LACK in CFA. Ten days after immunization, draining lymph node cells were prepared and incubated for 72 hr in the presence of medium alone or with the indicated concentrations of LACK peptide. Cytokine production was determined by ELISA for IFNγ as described in the legend to Figure 2. There was no detectable IL-4 or IL-5 in any of the samples analyzed.

RIP-TNFα mice produced significant amounts of IFNγ in response to LACK in vitro, while lymph node cells from RIP-LACK/RIP-TNFα mice failed to produce IFNγ (Figure 5). These data indicate that LACK-reactive T cells from RIP-LACK/RIP-TNFα mice are tolerized prior to immunization.

Coexpression of LACK and TNFα in Pancreatic β Cells Results in Increased Secretion of Th2-Like Cytokines by LACK-Specific TCR Transgenic T Cells

To monitor the effects of TNFα on LACK-specific immune responses in the absence of immunization, RIP-LACK/RIP-TNFα double-transgenic mice were crossed to TCR-LACK transgenic mice in which approximately 10% of peripheral $CD4^+$ T cells express a TCR transgene specific for a class II–restricted LACK peptide (158–173) (Brown et al., 1997). Purified $CD4^+$ cells from triple TCR-LACK/RIP-LACK/RIP-TNFα, double TCR-LACK/RIP-LACK and TCR-LACK/RIP-TNFα, and single TCR-LACK transgenic mice were stimulated in vitro with various concentrations of LACK peptide in the presence of syngeneic antigen-presenting cells (APCs) without exogeneously added cytokines. We found that $CD4^+$ T cells from triple-transgenic mice that expressed both LACK and TNFα in the pancreas produced less IL-2 but comparable amounts of IFNγ as compared to cells from double- or single-transgenic mice (Figures 6A and 6B). In contrast, cells from triple-transgenic mice produced more IL-4, IL-5, and IL-10 than those from single- or double-transgenic mice (Figures 6C–6E).

LACK-specific antibody responses were also monitored in the four groups of TCR transgenic mice by enzyme-linked immunosorbent assay (ELISA). In contrast to LACK-specific IgG1 antibodies, which were not detectable in any of the mice analyzed, we found high levels of LACK-specific IgG2a antibodies in the serum of TCR-LACK/RIP-LACK transgenic mice (Figure 6F). This spontaneous antibody response was not evident in triple TCR transgenic mice that express both LACK and TNFα in the pancreas (Figure 6F), a result that is broadly in agreement with the altered phenotype of $CD4^+$ T cells found in these mice.

Coexpression of TNFα and LACK in the Pancreas Leads to a Decrease in the Number of TCR Transgenic T Cells Exhibiting an Activated Phenotype

To determine how TCR transgenic T cells are modified when they encounter LACK in double-transgenic RIP-

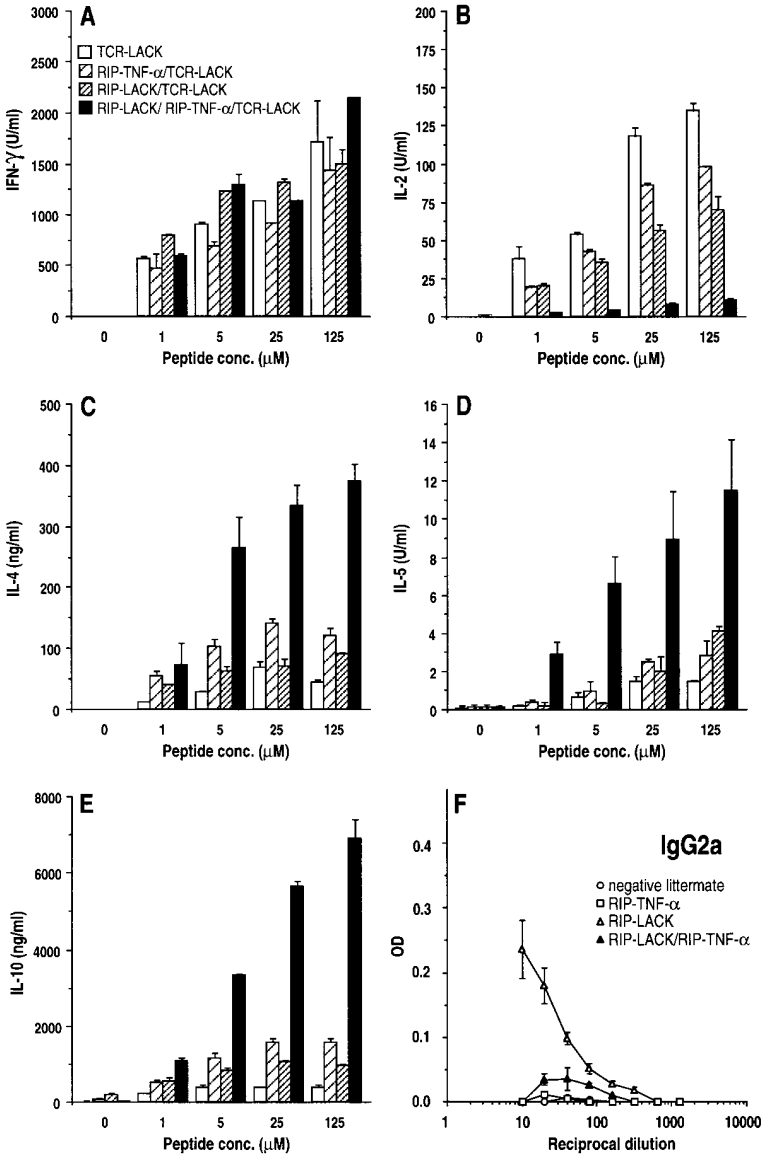

Figure 6. CD4+ TCR Transgenic T Cells from RIP-LACK/RIP-TNFα Mice Secrete More Th2-Like Cytokines but Less IL-2 Than Cells from Single-Transgenic Mice or Negative Littermates

CD4+ T cells were purified from the spleens of individual TCR-LACK/RIP-LACK, TCR-LACK/RIP-TNFα, TCR-LACK/RIP-LACK/RIP-TNFα, and TCR-LACK mice and incubated for 72 hr with syngeneic APCs in the presence of medium alone or with the indicated concentrations of LACK peptide without exogeneously added cytokines. Cytokine production was determined by ELISA for (A) IFNγ, (B) IL-2, (C) IL-4, (D) IL-5, and (E) IL-10. The levels of LACK-specific IgG2a in the serum of these mice was also measured by ELISA (F). No LACK-specific IgG1 was detected in these mice (data not shown).

LACK/RIP-TNFα mice, we used flow cytometry to analyze and compare the phenotypes of CD4+Vβ4+ T cells of age-matched and cohoused mice from the four groups. Although the total number of CD4+Vβ4+ T cells was not different among the four groups of mice (data not shown), we found that the proportion of CD4+Vβ4+ T cells exhibiting an activated large CD44hiCD62Llo phenotype was increased in RIP-LACK transgenic mice as compared to RIP-TNFα transgenic mice or negative littermates (Figure 7). Thus, the expression of LACK in the pancreas leads to the activation of LACK-reactive TCR transgenic T cells, a result that is in agreement with the spontaneous LACK-specific IgG2a antibody response found in these mice. In mice that expressed both TNFα and LACK in the pancreas, the proportion of CD4+Vβ4+ T cells exhibiting an activated phenotype was reduced, a phenomenon likely to be linked to the specific tolerance of T cell responses caused by the expression of TNFα.

Discussion

The role that TNFα plays in the development of spontaneous diabetes is still a matter of speculation. A number of reports have suggested that transgenic expression of TNFα in islet β cells favors the development of autoreactive T cells, which then incite a destructive autoimmune process. Thus, transgenic mice in which both TNFα and the costimulatory molecule B7 are expressed in pancreatic β cells develop spontaneous diabetes, whereas mice expressing only the B7 transgene do not progress from insulitis to diabetes (Guerder et al., 1994). In addition, double-transgenic mice in which both TNFα and the lymphocytic choriomeningitis virus GP antigen are expressed in islet β cells developed diabetes following infection with a recombinant GP–vaccinia virus, while single-transgenic mice expressing only the GP protein did not (Ohashi et al., 1993). Thus, in both these model systems the local expression of TNFα provides

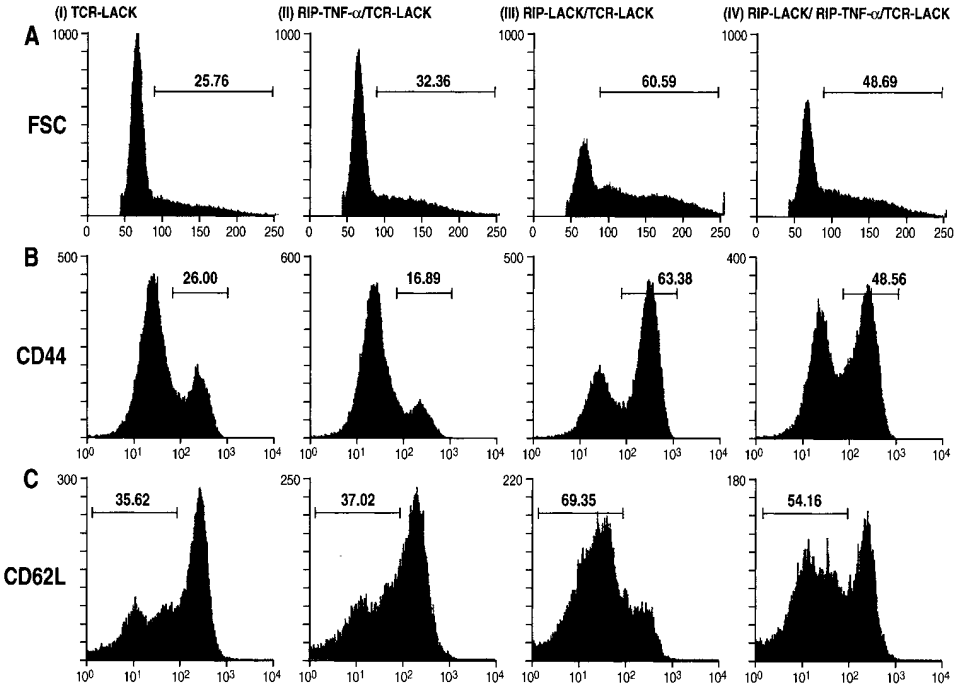

Figure 7. Flow Cytometric Analysis of TCR Transgenic $CD4^+$ T Cells in RIP-LACK/RIP-TNFα Mice

Cell suspensions were prepared from the spleens of the indicated mice and stained with fluorescent antibodies to CD4, Vβ4, and CD44 or CD62L before being processed for flow cytometric analysis. For each marker, typical flow cytometric patterns for (A) forward scatter, (B) CD44, and (C) CD62L are shown after gating on $CD4^+V\beta4^+$ cells. Numbers show the percentage of cells within the indicated gates.

an inflammatory environment that allows development of autoreactive T cells and subsequent progression to diabetes.

In apparent contrast with these results, Grewal et al. (1996) recently reported that RIP-TNFα mice generated on a NOD background developed spontaneous insulitis but failed to progress to a diabetic state, a process that correlates with a lack of spontaneous responsiveness to islet antigens that normally occurs in NOD mice. Although other mechanisms could be responsible for this phenomenon, it was proposed that the transgenic expression of TNFα in these mice did not allow efficient costimulation of self-reactive T cells, thus preventing the spontaneous T cell response to islet antigens that normally occurs in NOD mice.

To understand better how local expression of TNFα in islet cells can affect T cell responses to a defined islet antigen, we constructed RIP-LACK transgenic mice in which the expression of the L. major LACK antigen was restricted to pancreatic β cells. Because the RIP-LACK mice were fully capable of mounting LACK-specific antibody and T cell responses when immunized with LACK, we were able to determine how local expression of TNFα affects LACK-specific immune responses by crossing RIP-LACK mice to RIP-TNFα mice. In agreement with the report by Grewal et al. (1996), we found that double-transgenic mice expressing both LACK and TNFα in pancreatic islets exhibited an impaired ability to mount a LACK-specific T cell immune response. Both Th1 and Th2 compartments were affected, as suggested by the reduced numbers of IFNγ–, IL-4– and IL-5–producing cells found in the lymph nodes of these mice following immunization. The functional consequence of a lack of T cell help was observed on antibody responses: the amounts of LACK-specific IgG1 and IgG2a antibodies were greatly reduced in the double-transgenic RIP-LACK/RIP-TNFα mice compared to mice from the other three groups. What mechanisms are responsible for this phenomenon? The impaired ability of the double-transgenic mice to mount a LACK-specific T cell response following immunization suggests that LACK-reactive T cells have encountered the antigen, a process that then leads to active tolerization of these $CD4^+$ T cells. Adoptive transfer experiments using nude mice were in agreement with this conclusion and further supported the hypothesis that tolerization occured prior to immunization. Thus, local expression of TNFα in the pancreas does not merely prevent the activation of islet specific T cells, as previously suggested (Grewal et al., 1996), but leads to antigen specific tolerance.

Where do naive LACK-reactive cells encounter the antigen for the first time? Although the encounter could occur in the pancreas, it is unlikely that all cells reactive to LACK would traffic to this organ for tolerization. A second possibility is that the effect is mediated through APCs that migrate to the pancreas as a result of transgenic expression of TNFα, as has been shown in transgenic mice expressing lymphotoxin in pancreatic β cells (Kratz et al., 1996). These APCs would then capture cellular debris from β cells and present them in a tolerogenic form to the immune system, perhaps after having migrated to the thymus or to the spleen.

The use of TCR-LACK transgenic mice has allowed

us to investigate further the mechanisms by which local expression of TNFα in islet β cells leads to the development of tolerance to a defined pancreatic antigen. However, our experiments also uncovered further complexities concerning the effect of TNFα. Surprisingly, we found that the spleens of TCR transgenic mice that carried the RIP-LACK but not the RIP-TNFα transgene contained more $CD4^+V\beta4^+$ T cells exhibiting an activated phenotype than did the spleens from other TCR transgenic mice. This was in agreement with the spontaneous LACK-specific IgG2a antibody response found in the TCR-LACK/RIP-LACK mice and further suggested that these T cells were capable of providing help to B cells in vivo following activation. In agreement with a possible role of TNFα in the down-regulation of T cells responses, the proportion of cells expressing an activated phenotype in RIP-LACK/TCR-LACK mice was reduced in mice that also expressed the RIP-TNFα transgene. Expression of TNFα in the pancreas also curtailed the spontaneous LACK-specific IgG2a antibody response found in the TCR-LACK/RIP-LACK mice. Although $CD4^+$ T cells from TCR-LACK/RIP-LACK/RIP-TNFα transgenic were partially tolerized, as observed in their impaired ability to secrete IL-2 in response to antigenic stimulation, they also secreted higher levels of Th2-like cytokines such as IL-4, IL-5, and IL-10. Thus, local expression of TNFα in the islets of the TCR-transgenic mice favored the development of LACK-reactive Th2 cells. Because naive T cells do not express TNF receptors, it is unlikely that TNFα acts directly on these cells. This process may be explained by TNFα- mediated stimulation of production in vivo of IL-6, a cytokine that recently has been shown to drive Th2 development (Rincon et al., 1997). At this stage, however, we cannot demonstrate whether TNFα affects T cells directly or via the induction of other cytokines or effects on antigen presentation. Future experiments with TNF-receptor knockout mice should allow us to distinguish between these possibilities. Whatever the mechanism, a similar role for TNFα in the development of Th2 responses has previously been shown in an adoptive-transfer TCR transgenic model in which TNFα was used as an adjuvant, resulting in the specific production of IgG1 antibodies (Pape et al., 1997).

It is noteworthy that the biased Th2 responses found in triple-transgenic mice was not found in double-transgenic RIP-LACK/RIP-TNFα mice following immunization with LACK. A number of mechanisms may account for this observation. First, the capacity of TNFα to amplify a Th2 response may depend upon the frequency of antigen-reactive T cells, which is much higher in TCR transgenic mice than in mice with an unmanipulated T cell repertoire. Indeed, it has been shown that the polarization of Th1 and Th2 cells is dramatically affected by alterations in the ratio of antigen to responding T cells (Hosken et al., 1995). Alternatively, TCR-LACK transgenic mice exhibit, as do many other TCR strains, a large proportion of cells exhibiting an activated phenotype. Because activated cells, in contrast to naive cells, express TNFα receptors, it is possible that TNFα exerts a direct cytotoxic effect on these cells, possibly by inducing the preferential cell death of Th1-like cells.

Our data may help to explain the apparent dichotomy in the role of TNFα in the induction of diabetes. Thus, in models that have been shown to (Ohashi et al., 1993) or that probably (Guerder et al., 1994) involve the destruction of β cells through a $CD4^+$-independent process, TNFα accelerates the development of the disease process. However, we have demonstrated here that TNFα leads to the tolerance or immune deviation of $CD4^+$ T cells that are required for the diabetogenic process in NOD mice. Thus, while TNFα may participate in the development of diabetes once the process of β cell destruction has begun, our data suggest that the initial role of TNFα may be to prevent the induction of a destructive self-reactive response before this occurs. Indeed, such an hypothesis has been suggested but not demonstrated in studies in which recombinant TNFα or neutralizing antibodies have been administered to NOD mice (Yang et al., 1994). Both the arrest of the diabetic process at the insulitis stage in RIP-TNFα/NOD mice and the fact that RIP-TNFα mice are resistant to streptozotocin-induced diabetes (our unpublished data) also are in agreement with this hypothesis.

Experimental Procedures

Mice

BALB/c and BALB/cByJ-Nude mice were purchased from IFFA Credo and were used at 6–10 weeks of age. Mice were bred in our core Animal Facility and housed under specific pathogen–free conditions. RIP-TNFα (Picarella et al., 1993) and IE-LACK (Julia et al., 1996) transgenic mice have been described previously. TCR-LACK mice were produced by microinjection of genomic TCR constructs into (B10.S × C57BL/6) F1 fertilized eggs and implantation into pseudopregnant foster mothers according to standard procedures (Hogan et al., 1986) (Reiner et al., unpublished data). The rearranged α and β chain gene constructs were made from DNA derived from the T helper cell clone 9.1–2 (Scott et al., 1990), which is specific for the L. major LACK antigen (Mougneau et al., 1995) and uses Vα8 and Vβ4 TCR variable regions. RIP-LACK transgenic mice were generated by subcloning a truncated LACK cDNA into a modified RIP-Eα expression vector downstream of the rat insulin gene promoter (RIP) (Guerder et al., 1994) and by injecting this construct into (B10.S × C57BL/6) F1 fertilized eggs. Three transgene positive founders were indentified by screening mice by Southern analysis of tail DNA. Each founder was crossed to BALB/c mice, and offspring were monitored for LACK expression in pancreatic islets by RT-PCR. For this study, all mice were backcrossed at least five times on the BALB/c background. Pancreatic sections from RIP-TNFα mice bred to a BALB/c background for five generations showed massive insulitis, as previously reported for the same mice on a C57BL/6 background (Picarella et al., 1993). Double-transgenic mice were generated by breeding RIP-LACK mice to RIP-TNFα mice. Triple-transgenic mice were generated by breeding double RIP-LACK/RIP-TNFα mice to TCR-LACK mice. Mice were screened for RIP-LACK and RIP-TNFα transgenes by PCR analysis of tail DNA using the primers LM24 (ATCTGCTTCTCGCCGTCGCT) and LM25 (GCGTCGGAGATGGACCACAC), or TNF1 (ATTTGAGGGACGCTGT GGGCTCTT) and TNF2 (TGTGAGGGTCTGGGCCATAGAACTGAT), respectively. Tail DNA was prepared by proteinase K (Boehringer Mannheim) digestion, phenol/chloroform (GIBCO–BRL) extraction, and ethanol precipitation. A 466 bp (LM24 and LM25) PCR product was amplified from RIP-LACK mice and a 800 bp product from RIP-TNFα mice (TNF1 and TNF2). Mice were screened for the TCR transgene by flow cytometry of peripheral blood cells using the MAb KT4.1.

Reagents and Antibodies

LACK recombinant protein was produced in Escherichia coli and purified as described (Mougneau et al., 1995). LACK (amino acids

158–173, FSPSLEHPIVVSGSWD) and OVA peptides (amino acids 323–338, ISQAVHAAHAEINEAG) were purchased from Chiron Mimotopes. The following MAbs were used for cell purification or analysis: GK1.5, anti-CD4 (Dialynas et al., 1983); Mel-14, anti-CD62L (PharMingen, San Diego, CA); 7D4, anti-IL-2 receptor α (PharMingen); IM7, anti-CD44 (PharMingen); KT4.1, anti-Vβ4 (Tomonari et al., 1990); 11B11, anti-IL-4 (Ohara and Paul, 1985); R46A2, anti-IFNγ; and anti-mouse IgG2a and IgG1 (PharMingen). Recombinant mouse IL-2 and IL-5 were purchased from Genzyme (Cambridge, MA). Recombinant IFNγ was a kind gift from B. Stockinger (National Institute for Medical Research, London, UK), and recombinant IL-4 and IL-10 were generously provided by F. Y. Liew (University of Glasgow, Glasgow, UK).

RNA Analysis

RNA from different tissues was extracted by acid guanidinium-thiocyanate (Chomczynski and Sacchi, 1987), treated with DNAse I (Boehringer Manheim), reverse transcribed (GIBCO–BRL) into cDNA, and then amplified by 35 cycles of PCR using the primers LM4 (GGAATTCATTGCGGAGCTGACG) and LM16 (GGGTTCGAATTACT CGGCGTCGGAGATT), which resulted in a 150 bp product. Samples were analyzed by electrophoresis through a 2% agarose gel. HPRT (hypoxanthine phosphoribosyl transferase) primers were used for RNA from all tissues as a control for the efficiency of cDNA synthesis.

Immunization

Mice were immunized in each hind footpad with 50 μg of LACK or ovalbumin (Sigma) in CFA (1:1) (Sigma), and draining lymph node cells were harvested 10 days later.

Tissue Culture Medium

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), 1 mM sodium pyruvate, L-glutamine (2 mM), and 2-β-mercaptoethanol (50 μM) was used for all stimulations and assays.

Cytokine Release Assays

$1–2 \times 10^6$ lymph node cells from immunized mice were incubated in flat-bottom 96-well plates for 72 hours in the presence of various concentrations of LACK or OVA peptide. Supernatants were then harvested and analyzed for the presence of IFNγ, IL-2, IL-4, IL-5, and IL-10 by ELISA as previously described (McSorley et al., 1997). The cytokine content of each sample was established by comparison to a standard curve for the appropriate recombinant cytokines.

Cytokine ELISPOTs

To determine the frequency of antigen-reactive IL-4–, IL-5–, or IFNγ–producing T cells, an ELISPOT technique was used as described (Soldera et al., 1997). In brief, single-cell suspensions of lymph node cells from immunized mice were prepared and 1×10^7 cells incubated with 25 μM LACK or OVA peptide in 1 ml of complete DMEM. After culture for 72 hr, live cells were recovered by passage through Ficoll-Hypaque ($d = 1.083$) before being washed and transferred by serial dilution (10^4 to 5×10^5 cells/well) to 96-well microtiter plates (Millipore) that had been precoated with 4 μg/ml of capture MAbs, anti-IFNγ, anti-IL-4, or anti-IL-5 and blocked with DMEM/10% FCS. After 24 hr, cells were removed and spots were visualized using biotinylated detecting MAbs and avidin D peroxidase in conjunction with 3-amino-9-ethylcarbazole substrate (Sigma). Spots were counted under a dissecting microscope, and the frequency of antigen-specific cells was determined from the difference between the numbers of spots seen with and without antigen.

Antibody Responses

Antibody against LACK was measured by an ELISA method using 96-well plates coated with soluble Leishmania antigen as previously described (Xu et al., 1995). After blocking, serial dilutions of serum were added to the plates, and levels of L. major–specific IgG1 and IgG2a were determined using anti-mouse IgG1 or IgG2a antibodies.

Adoptive Transfer to Nude Mice

Spleen cells from RIP-TNFα, RIP-LACK/RIP-TNFα or IE-LACK mice were depleted of sIg^+ B cells by panning using sheep anti-mouse Ig serum (Cappel Laboratories). After washing, cells were injected into the tail vein of nude recipients.

Cytokine Production by $CD4^+$ TCR Transgenic T Cells

$CD4^+$ T cells from the spleen of TCR-LACK, TCR-LACK/RIP-LACK, TCR-LACK/RIP-TNFα, and TCR-LACK/RIP-LACK/RIP-TNFα transgenic mice were purified from individual mice by positive selection. In brief, spleen cells were incubated on ice with fluorescein isothiocyanate (FITC)–conjugated anti-CD4 followed by anti-FITC microbeads (Miltenyi Biotech) before selection using a magnetic cell sorter according to the manufacturer's intructions. All samples were at least 98% pure as assessed by flow cytometry after two serial passages through the columns. Purified $CD4^+$ T cells ($1–2 \times 10^5$) were incubated in round-bottom 96-well tissue culture plates with mitomycin C–treated spleen cells as APCs and various concentrations of LACK peptide. After 72 hr, supernatants were harvested and examined for the presence of cytokines.

Flow Cytometry Analysis

Single-cell suspensions were prepared from the spleens of transgenic mice, depleted of erythrocytes by hypotonic lysis, and stained at 4°C in phosphate-buffered saline containing 5% FCS. Fluorescein- and phycoerythrin-conjugated MAbs specific for mouse CD4, Vβ4, CD44, and CD62L were used for triple staining. Viable cells (10^6 cells per sample), gated by exclusion of propidium iodide–positive cells, were analyzed on a FACScan flow cytometer (Becton Dickinson).

Statistical Analysis

Significant differences between groups were evaluated using a two-tailed Student's test ($p < 0.05$).

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